Synopsis

Datura metel is a herbaceous plant found in almost all tropical parts of the world. It belongs to the family Solanaceae whose members, viz. *Duboisia, Atropa, Hyoscyamus* and *Datura* plants are known to produce tropane alkaloids- hyoscyamine and scopolamine which are most noted for their therapeutic use as anti-cholinergic agents. Since these alkaloids are produced in very low amounts in plants, alternative sources and methods of production for these alkaloids have been crucial in meeting the demands for these drugs. Endophytic fungi inhabiting a plant may have the potential to produce the same compounds as the host plants. The aim of the present study was to search for tropane alkaloid producing endophytic fungal isolates from *Datura metel*.

Eighteen endophytic fungi were isolated from various tissues of *Datura metel* and screened for the presence of three tropane alkaloid biosynthetic genes- putrescine N-methyltransferase (PMT), tropinone reductase I (TRI) and hyoscyamine 6β-hydroxylase (H6H) using PCR-based screening approach. Six endophytic fungal isolates were found to possess the PMT, TR1 and H6H genes. The fungi were identified using molecular taxonomy as *Colletotrichum boninense, Phomopsis sp., Fusarium solani, Colletotrichum incarnatum, Colletotrichum siamense* and *Colletotrichum gloeosporioides* and the identity was confirmed using colony and spore morphology. The production of tropane alkaloids hyoscyamine and scopolamine by the fungi has been ascertained using various techniques like TLC, HPLC and ESI-MS/MS by comparison with the authentic reference standards. The amount of tropane alkaloids produced by all six fungi in liquid cultures was quantified using HPLC analysis. Among the six tropane alkaloid-producing fungi *Colletotrichum incarnatum* gave the highest yields of hyoscyamine and scopolamine which were 3.906 mg/L and 4.13 mg/L, respectively.

With an aim to characterize the tropane alkaloid biosynthetic genes in these fungi, the PMT gene was isolated from five of the endophytic fungi- *Colletotrichum boninense, Fusarium solani, Colletotrichum incarnatum, Colletotrichum siamense and Colletotrichum gloeosporioides* for the first time and the sequence analysis showed high homology (98%) to the *Datura metel* PMT cDNA. The gene was found to be devoid of introns in the fungi. Further phylogenetic analysis of the full length PMT sequence from the fungi strongly supports the hypothesis of horizontal gene transfer between the host plant and endophytic fungi.

For further in detail characterization of fungal PMT, the *Colletotrichum boninense* PMT gene was taken as a representative. *Cb*PMT gene was cloned in pRSET A expression vector and heterologously expressed in *E. coli* and biochemically characterized. For optimal yield of soluble protein upon heterologous expression different conditions such as IPTG concentration, temperature and time post induction were optimized. Optimal yield was obtained by inducing the culture by 0.25 mM IPTG once it had reached and O.D. of 0.6 and incubating at 37°C for 3 h. The recombinant *Cb*PMT enzyme expressed as histidine tagged fusion protein was purified using Ni-NTA affinity chromatography. Gel elution studies were carried out to determine molecular weight of the protein and it was found that the protein exists as a homodimer in solution with some amount also present as a monomer. Catalytic activity of the purified recombinant enzyme was studied for its dependence on both substrates putrescine as well as S-adenosylmethionine (SAM). The Km and Vmax values for putrescine were found to be 464 μM and 18.55 nkat/mg, respectively, while those for S-adenosylmethionine were found to be 628 μM and 18.63 nkat/mg, respectively. Optimum temperature for activity was found to be 37°C and optimum pH range was found to be 8-9.

Fluorescence spectroscopy was used to study the binding affinity of both the substrates to the enzyme. Fluorescence quenching data for each substrate was analysed by using a nonlinear regression curve fit and K_d values were found to be 0.309 mM for putrescine and 0.118 mM for SAM, respectively. Circular dichroism spectrum of the enzyme indicated a pattern typical for alpha helix in the secondary structure. Binding of either substrate led to increase in ellipticity of the protein. Fluorescence quenching studies with collisional quenchers- acrylamide, potassium iodide, and cesium chloride indicated that

the native protein is folded in a conformation that allows tryptophan residues to be accessible for quenching. The fraction of tryptophan residues (f_a) accessible for quenching by acrylamide (1.06) was found to be higher than that for potassium iodide (0.54) while that cesium ions was the least (0.38). The neutral quencher acrylamide could access all the tryptophans meaning that none of tryptophans are completely buried inside hydrophobic cores. the differential accessibility to the charged quenchers, however, indicates that more of the tryptophans are surrounded by positively charged amino acids.

The unfolding of the protein was studied with the aid of chaotropic agents guanidine-HCl and urea and thermodynamic parameters were determined. The denaturant m-values were found to be 2.313 kcal/mol/M for Gdn-HCl and 2.345 kcal/mol/M for urea respectively. The free energy of unfolding was estimated to be 2.635 kcal/mol for Gdn-HCl and 4.630 kcal/mol for urea. Since no reports are available about the thermodynamics of folding and unfolding of PMT from any plant source, this study contributes towards the understanding of protein stability.

Although a lot of reports are available on the biochemical characterization of PMT from different plant sources, the crystal structure of PMT is not yet available. In the current work, homology based modelling studies on *Cb*PMT were carried out to get some idea about the protein tertiary structure. Homology based modelling studies showed that a significant amount of protein is present as α-helices which are present on the surface while the β-sheets are present in the interior of the protein. Each monomer of the protein is capable of binding both the substrates and hence the dimerization property of the enzyme could be a purely structural one leading to more stability and solubility of the protein.

In conclusion, this study has shown for the first time that endophytic fungi have significant potential to be used for tropane alkaloid production and six such fungal strains have been identified. Although the production of tropane alkaloids by endophytic fungi is not very high, it can be scaled up by over-expressing the biosynthetic gene putrescine N-methyltransferase in the highest producer- *Colletotrichum incarnatum* to further increase the yield. These endophytic fungi have significant potential to be applied in fermentation technology to meet the demands for these drugs economically.